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Processing and secretion of rat α_1-microglobulin-bikunin expressed in eukaryotic cell lines

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Abstract The precursor protein α_1-microglobulin-bikunin was cleaved to the same degree whether expressed in CHO cells or in mutated CHO cells, RPE.40 cells, suggested to lack a functional form of the intracellular protease furin. Thus, α_1-microglobulin-bikunin probably is not cleaved in vivo by furin. However, simultaneous overexpression of the precursor and furin in COS, CHO and RPE.40 cells increased the cleavage, suggesting that compartmentalisation and concentrations of protease and precursor are important for the cleavage, besides the in vitro specificity. Expression of α_1-microglobulin and bikunin alone gave different protein patterns on SDS-PAGE as compared to expression of the precursor and subsequent cleavage, suggesting that the precursor protein is important for the post-translational handling of α_1-microglobulin and bikunin.

Key words: Furin; Precursor; COS cell; CHO cell; RPE.40 cell; Post-translational modification

1. Introduction

Rat α_1-microglobulin is a brown-coloured 28 kDa glycosylated plasma protein with immunoregulatory properties. α_1-microglobulin is a member of the lipocalin superfamily, which comprises nearly twenty different proteins with a predicted three-dimensional structure of two anti-parallel β-sheets surrounding a hydrophobic pocket [1].

The mRNA for α_1-microglobulin also codes for bikunin [2], and an α_1-microglobulin-bikunin precursor protein is synthesized in rat liver cells [3], and cleaved late in the Golgi apparatus [4]. Bikunin is the light chain of the high molecular weight plasma proteins inter-α-inhibitor, formerly inter-α-trypsin inhibitor [5], and pre-α-inhibitor [6], and responsible for the anti-proteolytic activities of these proteins. After cleavage of the precursor, α_1-microglobulin is secreted in free form into the bloodstream [4]. Bikunin is glycosylated and sulphated, most likely before cleavage of the precursor [7], and the resulting glycosaminoglycan crosslinks the inhibitor complexes [8]. In the precursor protein, the α_1-microglobulin and bikunin parts are separated by a tetrapeptide conforming to a consensus sequence with basic amino acids in positions −4, −2 and −1 relative to the cleavage site [9,10]. This sequence is found in several different proproteins, such as complement and coagulation factors, receptors, virus envelope proteins and growth factors [10], and is thought to be a recognition site for an intracellular endoprotease family, including furin [11] (also given the systematic name PACE1) as the most well characterized.

Two questions concerning the processing of the α_1-microglobulin-bikunin precursor were addressed in this article. First, the role of furin in the processing was investigated by expressing DNA coding for α_1-microglobulin-bikunin alone or together with DNA coding for furin in cell lines with no endogenous α_1-microglobulin-bikunin production. Secondly, the co-expression of α_1-microglobulin and bikunin as a precursor protein is enigmatic as the two proteins do not have any apparent functional connection. In an effort to find the reasons for the co-expression, DNA fragments encoding either the full precursor protein or the α_1-microglobulin- or bikunin-parts alone were expressed in the same cell system, and the protein products compared.

2. Experimental

2.1. Materials

CHO-K1 (referred to as CHO) and RPE-40 cells were generous gifts from Dr. T. Moehring, Vermont Cancer Center, Burlington, USA. Restriction enzymes *Sac*I and *Xho*I and Endoglycosidase F were purchased from Boehringer Mannheim, Germany and *Sal*I, *Xba*I, *Sma*I, *Bam*HI, DNA polymerase (Klenow fragment), T4 DNA ligase, Lipofectin, RPMI 1640, Optimem and foetal calf serum (FCS) from Gibco BRL, Gaithersburg, USA. Taq polymerase and *f*mol sequencing kit was obtained from Promega, Madison, USA. pBluescript containing human furin was a gift from Chiron Co., Emeryville, USA. Primers were purchased from Biomolecular Resource Facility, University of Lund, Sweden. Rabbit anti-rat α_1-microglobulin [12] and goat anti-rabbit IgG [13] were prepared as described. Chemicals were from Merck, Darmstadt, Germany, if not indicated otherwise.

2.2. DNA constructs

DNA encoding α_1-microglobulin-bikunin, α_1-microglobulin or bikunin, including the natural signal peptide of α_1-microglobulin, were constructed (Fig. 1) by the polymerase chain reaction (PCR) technique, using a rat liver library (Clontech Lab., Palo Alto, USA) as a template source and primers containing suitable restriction enzyme sites for ligation in the pSVL SV40 late promotor expression vector (Kabi-Pharmacia AB, Uppsala, Sweden). The primers used for α_1-microglobulin-bikunin amplification was primer 1 (AAGTCTCTAG AATGCAGGGT CTCGGGCCCT GTT) and primer 2 (CGACGAGCTC TCATCTTGCA AATGAAGTGG ACTC), for α_1-microglobulin amplification primer 1 and primer 3 (AAGTCGAGCT CTCAACTGCG TGTTAGCTCC TCGTAC) and for bikunin with signal sequence primer 4 (CTGCCTGCCT CACTTTGAAG GCTGCAGTGC TGCCCCAAGA GAATGA) and primer 2 in the first step and primer 5 (AATCTAGAAT GCAGGGTCTC GGGGCCCTGT TCTTGTTGCT GACTGCCTGC CTCACTTTGA AGGCTGCAGT GCT) and primer 2 in the second step. The PCR conditions were as follows: 94°C for 3 min, 25 cycles with 50°C for 1 min, 72°C for 1 min and 94°C for 30 s, and ending with 72°C for 10 min. The products were purified by 1.0% agarose gel electrophoresis, where the bands of interest were cut out and the agarose slices centrifuged on top of dimethyldichlorosilane-

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Abbreviations: FCS, foetal calf serum; CHO, Chinese hamster ovary; RPE, resistance to *Pseudomonas* exotoxin A; EMEM, Earle's minimum essential media.

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treated glass wool (Alltech, Deerfield, USA) at 4,000 × *g* for 10 min. To faciliate restriction enzyme cleavage, the PCR-products were made blunt-ended by treatment with DNA polymerase (Klenow fragment) and, after heat inactivation at 68°C, cut with restriction enzymes *Xba*I and *Sac*I. The fragments were ligated in pSVL, which previously had been cut with the same restriction enzymes. Ligation was performed with T4 DNA ligase using an insert/vector ratio of 3/1(molar) for 1 h in 23–26°C. pBluescript furin was cleaved by the restriction enzyme *Sal*I and made blunt-ended with the Klenow fragment. The product was cleaved by *Xba*I and the furin fragment was ligated, as above, into pSVL which had been cut by *Xba*I and *Sma*I. Unless otherwise indicated, protocols from Maniatis et al. [14] were used. All DNA constructs except furin were sequenced after insertion in pSVL using the *f*mol sequencing kit according to the dideoxy method [15].

2.3. Expression of α_1-microglobulin-bikunin

COS cells were grown on an 8.5 cm Petri dish (NUNC, Roskilde, Denmark) in RPMI 1640 medium supplemented with 10% FCS. The adherent cells were trypsinated after reaching confluency, washed once with PBS and transfected with pSVL containing DNA coding for α_1-microglobulin, bikunin and α_1-microglobulin-bikunin with or without pSVL coding for furin (100 pmol of each plasmid) by electroporation (Biorad, Hercules, USA) at 500 μF and 0.3 kV according to the manufacturer. All transfections described were done at least in duplicate. The cells were immediately sown out in 8.5 cm dishes in RPMI 1640 supplemented by 10% FCS and left at 37°C for 48 h.The medium was changed to Optimem and the cells and medium were harvested after 24 h. The cells were solubilized with 5 ml 0.1 M Tris-HCl (pH 8.0), 5 mM NaEDTA, 0.15 M NaCl, 0.1 mM *N*-ethylmaleimide, 10 mg Triton X-100/ml, 1 μg pepstatin/ml, 5 μg antipain/ml and 10 μg leupeptin/ml. For the $^{35}SO_4$-labelling of bikunin, the cells were transfected as above, sown out and four days later washed with EMEM low sulphate medium (Nordvacc, Sweden) also containing leucine 52 mg/l and then incubated for 24 h in this medium supplemented with 10% FCS and 0.1 MBq $^{35}SO_4$/ml (Amersham Life Science, Amersham, England). CHO and RPE.40 cells were grown on 8.5 cm Petri dishes in RPMI 1640 medium. pSVL coding for α_1-microglobulin-bikunin with or without pSVL coding for furin were transfected to the cells on the dish by adding a mixture of the plasmids (20 pmol) with 30 μl Lipofectin and 3 ml Optimem. 3 ml Optimem supplemented with 10% FCS were added 6 h after transfection. The medium was changed 30 h after transfection and the cells and medium harvested 72 h after transfection.

2.4. Affinity chromatography

Media and cell solubilisates were purified on a column packed with monoclonal anti-α_1-microglobulin Sepharose. The anti-rat α_1-microglobulin, designated BN11.6, was prepared and purified as described [16] and 5 mg were immobilized to 1 ml CNBr-activated Sepharose [17] according to the description supplied by Kabi-Pharmacia AB, Uppsala, Sweden. The columns were eluted with 0.1 M glycine-HCl, pH 2.2.

2.5. Analysis of α_1-microglobulin and bikunin

α_1-microglobulin (30 μg/ml), purified by affinity chromatography, was treated with Endoglycosidase F (1 mU/μg α_1-microglobulin) for 48 h at 37°C as described previously [4]. α_1-microglobulin and goat anti-rabbit IgG were labelled with ^{125}I (Svensk Radiofarmaka, Stockholm, Sweden) by chloramin T-oxidation [18] to a specific activity of 0.3–1.0 MBq/μg. Proteins were separated by 12% SDS-PAGE according to Laemmli [19], transferred to Immobilon membranes [20] (Millipore, Bedford, USA) and blotted with rabbit polyclonal anti-rat α_1-microglobulin antibodies and ^{125}I-goat anti-rabbit IgG as described [21]. Detection of proteins were done by autoradiography at −70°C using Kodak X-Omat S films (Kodak, Rochester, USA), or in a Fujix BAS

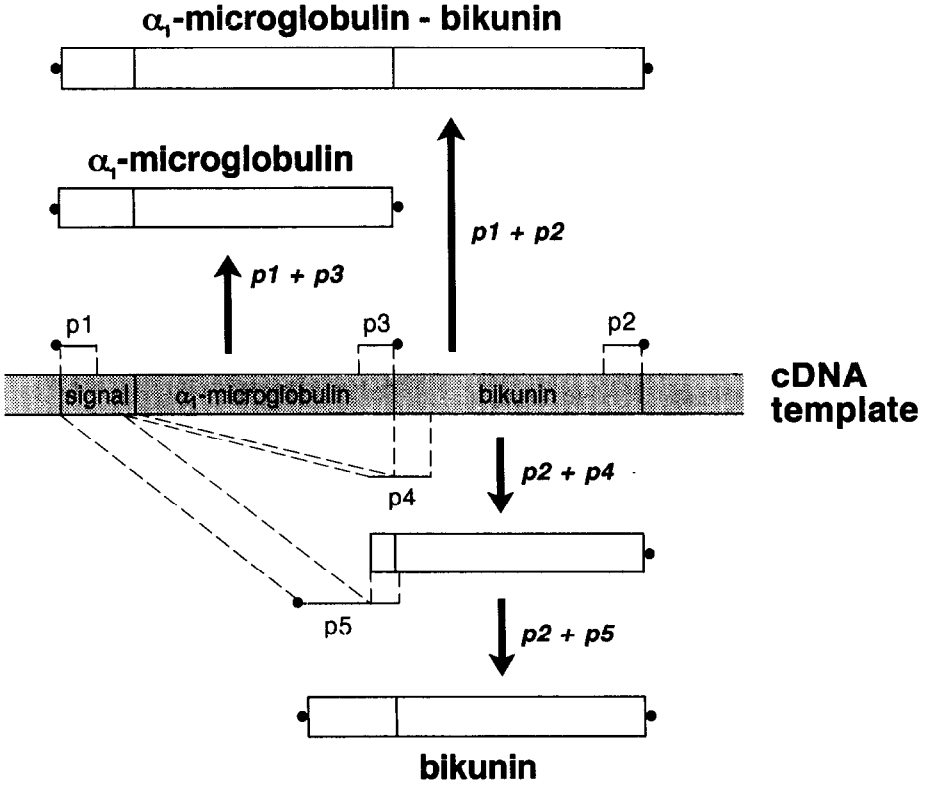

Fig. 1. DNA constructs. Different α_1-microglobulin- or bikunin-encoding DNA constructs (empty bars) were produced from a rat liver cDNA library (hatched bar) using PCR (arrows) and five different primers (unbroken lines) containing sequences from various sites of the α_1-microglobulin-bikunin DNA and restriction enzyme sites (dots). α_1-microglobulin was produced with primer 1, which was coding for a *Xba*I site and the 5′ end of the signal sequence of α_1-microglobulin, and primer 2, with a *Sac*I site and the 3′ end of bikunin. α_1-microglobulin was produced using primer 1 and primer 3, coding for the 3′ end of α_1-microglobulin, a stop codon and a *Sac*I site. Bikunin was produced using primer 4, which codes for the 3′ part of the natural signal sequence of the precursor and the 5′ part of bikunin, and primer 2. This product was purified and then used in a reaction with primer 5, coding for most of α_1-microglobulin's signal sequence and primer 2.

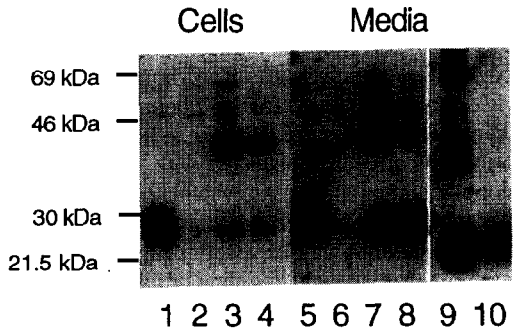

Fig. 2. Expression of α_1-microglobulin-bikunin forms in COS cells. COS cells were transfected with plasmids carrying DNA inserts coding for α_1-microglobulin, bikunin, α_1-microglobulin-bikunin or furin and medium was collected 72 h later. The cells were washed with PBS and solubilised with Triton X-100. Media and cell solubilisates were applied to an anti-rat α_1-microglobulin Sepharose column. The eluates were separated by SDS-PAGE and blotted with anti-rat α_1-microglobulin. Cell solubilisate from the COS cells after α_1-microglobulin transfection is shown in lane 1; cell solubilisate after bikunin transfection in lane 2; cell solubilisate after α_1-microglobulin-bikunin transfection in lane 3; cell solubilisate after α_1-microglobulin-bikunin and furin transfection in lane 4. Medium from the COS cells after α_1-microglobulin transfection is shown in lane 5; medium after bikunin transfection in lane 6; medium after α_1-microglobulin-bikunin transfection in lane 7; medium after α_1-microglobulin-bikunin and furin transfection in lane 8. Medium after α_1-microglobulin-bikunin transfection and Endoglycosidase F treatment in lane 9; medium after α_1-microglobulin transfection and Endoglycosidase F treatment in lane 10.

2000 Bio-imaging analyzer (Fuji films Co., Japan). $^{35}SO_4$-containing samples were separated under non-denaturing conditions on a 4–27% acrylamide gradient electrophoresis [22]. The gel was washed in water, soaked in 1.3 M sodium salicylate, dried and analysed by fluorography [23]. α_1-microglobulin concentrations were determined by a RIA procedure previously described [24], based on a competitive binding of labelled and unlabelled rat α_1-microglobulin to polyclonal rabbit anti-α_1-microglobulin, followed by precipitation of immune complexes with 10% polyethylene glycol 6000.

3. Results

3.1. DNA construction

DNA fragments coding for α_1-microglobulin-bikunin, α_1-microglobulin and bikunin were constructed as described in Fig. 1. All three DNA fragments contained the code for the natural signal sequence of α_1-microglobulin-bikunin. The α_1-microglobulin encoding fragments also included the sequence R-A-R-R situated N-terminally of the cleavage site. These constructs and DNA coding for furin were ligated into pSVL plasmids. The plasmids were subjected to restriction enzyme cleavage and agarose electrophoresis and the sizes of the DNA fragments were the expected from inserts adequately incorporated (not shown). All PCR constructs were sequenced and found to be identical to the previously published sequence [3].

3.2. Expression of α_1-microglobulin-bikunin in COS cells

To determine if furin can cleave the α_1-microglobulin-bikunin precursor protein under natural conditions, α_1-microglobulin-bikunin was expressed in COS cells, with or without concomitant expression of furin. SDS-PAGE and anti-α_1-microglobulin blotting demonstrated the presence of at least two high molecular mass forms (40 and 42 kDa) and two low molecular mass forms (28 and 30 kDa) in the cell lysates (Fig. 2, lanes 3 and 4) and media (lanes 7 and 8). As shown previously, the 40 and 42 kDa forms represent the precursor protein α_1-microglobulin-bikunin with one or two N-linked oligosaccharides, and the 28 and 30 kDa forms probably correspond to non-sialylated or sialylated free α_1-microglobulin which were present in rat liver cells as 26 and 28 kDa, respectively [4]. Less of the precursor protein and more free α_1-microglobulin was seen when furin was co-expressed with α_1-microglobulin-bikunin in the cells (lane 4) and media (lane 8), suggesting that furin is capable of cleaving the precursor with free α_1-microglobulin as one of the products. Substantial amounts of free α_1-microglobulin also appeared when α_1-microglobulin-bikunin was expressed without furin (lanes 3 and 7), suggesting that an endogenous protease with specificity for α_1-microglobulin-bikunin is present in the COS cells. A 65 kDa band and a heterogeneous staining between 40 and 65 kDa were also observed when α_1-microglobulin-bikunin was transfected without furin. Finally, a 50 kDa band was seen in all lanes as part of the background.

3.3. Expression of α_1-microglobulin in a protease deficient cell line

A CHO-derived cell strain called RPE.40, which is unable to cleave viral membrane glycoprotein precursors [25], probably due to a mutation in the furin gene, was used for expression of α_1-microglobulin-bikunin alone or together with furin. For both CHO and RPE.40 cells, transfection with α_1-microglobulin-bikunin yielded both the α_1-microglobulin-bikunin precursor and free α_1-microglobulin (Fig. 3, lanes 2 and 4), suggesting that enzymes capable of cleaving α_1-microglobulin-bikunin are present in both cell lines. The degree of cleavage, i.e. the ratio of precursor to free α_1-microglobulin, was approximately the same for the two cell lines, indicating that the mutation events in the RPE.40 cells do not involve the enzymes cleaving α_1-microglobulin-bikunin. Transfection of α_1-microglobulin-bikunin together with furin gave a higher degree of cleavage in

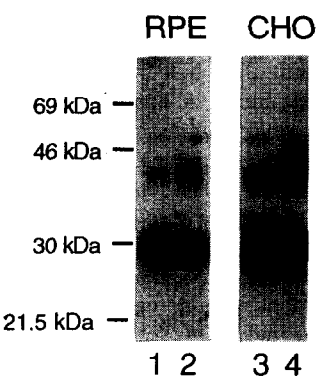

Fig. 3. Expression of α_1-microglobulin-bikunin with and without concomitant expression of furin in RPE.40 and CHO cells. The experiment was essentially performed as described for Fig. 2. Lane 1 represent medium from RPE.40 cells transfected with α_1-microglobulin-bikunin and furin, lane 2 medium from RPE.40 cells transfected with α_1-microglobulin-bikunin, lane 3 medium from CHO cells transfected with α_1-microglobulin-bikunin and furin, lane 4 medium from CHO cells transfected with α_1-microglobulin-bikunin.

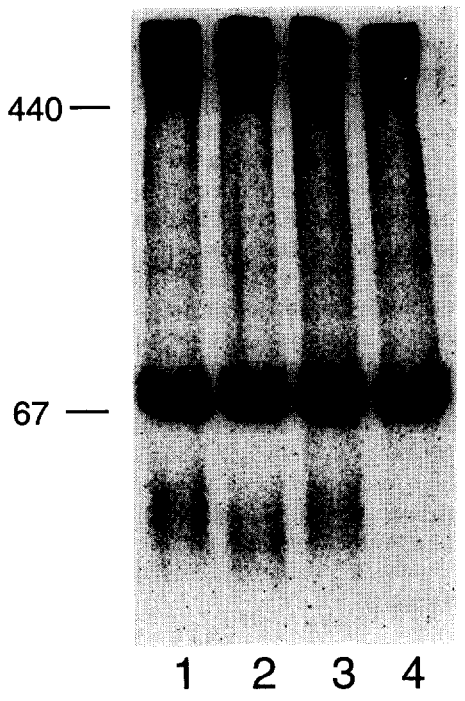

Fig. 4. Expression of $^{35}SO_4$-containing proteins in COS cells. COS cells were transfected with plasmids carrying DNA inserts coding for α_1-microglobulin-bikunin, furin, α_1-microglobulin or bikunin and cultivated in medium containing $^{35}SO_4$. Twenty-four hours later, medium was collected, and the cells washed with PBS and solubilised with Triton X-100. The media were separated by 4–27% acrylamide gradient electrophoresis, the gel treated with sodium-salicylate and dried. Lane 1 represents medium from COS cells transfected with α_1-microglobulin-bikunin and furin, lane 2 medium from COS cells transfected with bikunin, lane 3 medium from COS cells transfected with α_1-microglobulin-bikunin and lane 4 medium from COS cells transfected with α_1-microglobulin.

both cell types (lanes 1 and 3), again suggesting that furin is able to cleave the α_1-microglobulin-bikunin precursor.

3.4. *Expression of α_1-microglobulin or bikunin*

Transfection of DNA encoding the α_1-microglobulin part yielded similar amounts of free α_1-microglobulin as transfection with DNA encoding the whole α_1-microglobulin-bikunin precursor (typically around 0.6 μg/ml), as determined by RIA. However, when α_1-microglobulin was expressed alone a different protein band pattern was seen (Fig. 2, lanes 1 and 5). Transfection with the bikunin part alone yielded no α_1-microglobulin (Fig. 2, lanes 2 and 6).

To investigate the role of carbohydrates in the multiple forms of free α_1-microglobulin, media from COS cells after expression of α_1-microglobulin-bikunin or α_1-microglobulin without bikunin were treated with Endoglycosidase F, removing N-glycosides. After SDS-PAGE, the molecular masses of the α_1-microglobulin bands were reduced, but several different forms were still seen (Fig. 2, lanes 9 and 10). This suggests that the multiple forms of α_1-microglobulin are not caused by N-glycosides.

Bikunin can be seen in the crude culture medium of rat hepatocytes as a $^{35}SO_4$-containing broad band with a molecular mass of 35–45 kDa upon SDS-PAGE, due to the addition of a sulphated, heterogeneous glycosaminoglycan to the bikunin peptide [26]. Transfection of COS cells with DNA encoding α_1-microglobulin-bikunin with ot without furin or bikunin alone yielded a similar sulphated and heterogeneous molecule, most likely free bikunin (Fig. 4, lanes 1–3). This band was not present in control cells transfected with DNA encoding α_1-microglobulin alone (lane 4). As evident, expression of bikunin alone (lane 2) gave a smaller molecule than expression of α_1-microglobulin-bikunin with or without furin, suggesting a difference in post-translational handling of bikunin. Treatment of the culture media (containing bikunin) with chondroitinase [26] resulted in the disappearance of the broad $^{35}SO_4$-containing bands (not shown).

The effects of free intracellular bikunin, a protease inhibitor, on the protein synthesis in the cells was investigated by expressing bikunin alone in the presence of [^{35}S]methionine. After SDS-PAGE of the medium, no difference was seen in protein secretion (not shown). Furthermore, the COS cells, regardless of the identity of the transfected plasmids, appeared identical under the microscope.

4. Discussion

The cleavage of the α_1-microglobulin-bikunin precursor by the proprotein processing enzyme furin was examined in this work. Furin, a homologue to the yeast protease Kex2, was the first mammalian enzyme shown to have a cleavage specificity for intracellular proproteins with cleavage sites consisting of several basic amino acid residues also found in the α_1-microglobulin-bikunin precursor. Furin, which has a ubiquitous expression, is thus a strong candidate for the cleavage of the α_1-microglobulin-bikunin precursor in liver cells.

The α_1-microglobulin-bikunin precursor was successfully expressed and partly cleaved into its constituents α_1-microglobulin and bikunin in the cell lines CHO and RPE.40. The RPE.40 cells, which are mutated CHO cells, were shown to be unable to cleave provirus proteins, a function which could be restored by transfection with furin-encoding DNA [25]. A very similar processing defect was discovered in LoVo cells, which are derived from a human colon carcinoma, and in these cells a mutation of furin was demonstrated to be the cause of the defective proprotein processing [27]. This suggests that furin is also deactivated in the RPE.40 cell line, whereas the enzyme is intact in the CHO cells. The results of this work showed that there is no difference in the degree of cleavage of α_1-microglobulin-bikunin between the two cell lines (Fig. 3, lanes 1 and 3). The most likely conclusion of this experiment is that furin is not the α_1-microglobulin-bikunin cleaving enzyme in these cells.

Furin- and α_1-microglobulin-bikunin-encoding DNA were expressed in COS cells, CHO cells and RPE.40 cells. The results clearly showed an increased cleavage of the precursor as compared to expression of α_1-microglobulin-bikunin alone (Fig. 2 and 3). This shows that furin is *capable* of cleaving α_1-microglobulin-bikunin, even if the precursor is not a natural substrate of this enzyme. Overexpressed furin was recently shown to have a broader specificity than endogenous furin [28]. The increased intracellular cleavage of α_1-microglobulin-bikunin could be explained by an over-expression or an unnatural compartmentalisation of α_1-microglobulin-bikunin and furin in these in vitro expression systems, suggesting that these factors are of importance besides the specificities of the processing

enzymes in the regulation of the cleavage of various proproteins. Other proteases with similiar specificity as furin [29–31], which could be candidates for cleavage of α_1-microglobulin have been discovered. But no attempt to differentiate between these endoproteases has been done in this work.

Expression of α_1-microglobulin without bikunin in COS cells lead to the secretion of free α_1-microglobulin with different molecular masses than produced after expression of the full-length α_1-microglobulin-bikunin precursor. The code for the cleavage peptide located between α_1-microglobulin and bikunin in the precursor, R-A-R-R, was included in the DNA fragment encoding α_1-microglobulin without bikunin. It has been shown previously that the three C-terminal amino acid residues, A-R-R, have been removed in mature α_1-microglobulin [32]. One possibility is therefore that this peptide is eliminated to a much lower degree if free α_1-microglobulin is produced by direct translation instead of cleavage from the α_1-microglobulin-bikunin precursor. Treatment with Endoglycosidase F (Fig. 2), which removes N-glycosides, could not eliminate the different appearance on SDS-PAGE between α_1-microglobulin expressed alone and as the precursor. An alternative possibility is therefore that the protein is also O-glycosylated, and that this process depends on the presence of the precursor α_1-microglobulin-bikunin.

Interestingly, expression of bikunin without α_1-microglobulin gave a smaller molecule than expression of the whole α_1-microglobulin-bikunin precursor (Fig. 4). The difference was not caused by the presence of uncleaved sulphated α_1-microglobulin-bikunin since no precursor was present after co-expression of furin (lane 2), and the $^{35}SO_4$-activity was not removed by anti-α_1-microglobulin affinity chromatography (not shown). This observation thus suggests that the glycosylation and sulphation of bikunin, and perhaps also the cross-linking of bikunin to the heavy chains of pre- and inter-α-inhibitor which involves the sulphated glycosaminoglycan of bikunin [8], are regulated by the presence of the α_1-microglobulin part of the precursor.

The reason for the co-expression of α_1-microglobulin and bikunin is not known. The two proteins do not have any apparent functional connection as mature plasma proteins. It is possible that the explanation instead can be found in a functional interaction between the proteins before secretion. One such explanation is that the co-translation is needed to achieve a correct translation or secretion of either α_1-microglobulin and bikunin or both. However, the results of this work indicate that this is not the case since both proteins were translated and secreted to approximately the same level when expressed alone as when expressed via the precursor. Another possibility is that the co-translation ascertains a correct post-translational processing of either protein. Indeed, the results indicate a difference in glycosylation or exoprotease processing of α_1-microglobulin and glycosylation/sulphation of bikunin, when expressed alone.

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